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Carboxymethylated hemoglobin is more stable than oxy hemoglobin during some conditions of storage at various temperatures. Collaborative studies with Dr. John Hess and his colleagues at the Letterman Army Institute of Research indicated that carboxymethylated hemoglobin was cleared from the circulation of rats with a half time of 42 minutes. The pseudo-first order plot indicates that a homogeneous population of molecules was present. In addition, there were no adverse pathological findings.

Cross-linking agents are being evaluated to produce derivatives of molecular weight 64,000 without higher molecular weight species.

Like carboxymethylation, acetylation at the DPG site is another procedure to bring about a lowered oxygen affinity of hemoglobin. This approach has been explored with the site-specific acetylating agent and the results have been correlated with the effects of DPG and chloride on hemoglobin. In addition, the effect of chloride on the pH of phosphate-buffered saline has also been evaluated.

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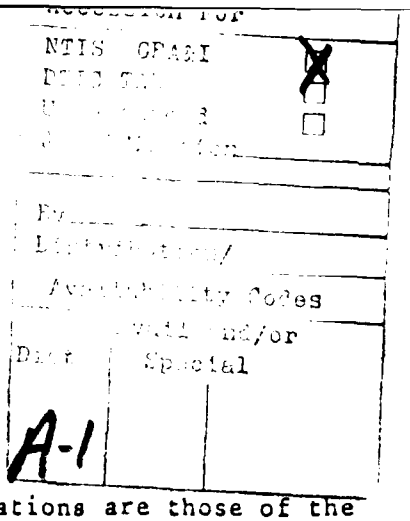

FOREWORD

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N.A. In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N.A. For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

N.A. In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

James M. Manning 9/2/89
PI Signature Date

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INTRODUCTION AND BACKGROUND

Our emphasis has been on two major objectives. We have determined the properties of the carboxymethyl derivative of hemoglobin to determine whether they are consistent with its possible future use as a blood substitute. We have evidence that this derivative is analogous to that formed in the reaction of hemoglobin with CO_2. This natural product results in a very low oxygen affinity for hemoglobin, which is a very desirable property for a blood substitute. The second major objective of our research has been to cross-link this hemoglobin derivative in a manner that will maintain the essential functional properties of hemoglobin.

The covalently attached carboxymethyl group also mimics another modulator of hemoglobin function, i.e., the chloride anion. Small inorganic anions such as chloride lower the oxygen affinity of hemoglobin to a significant degree (1). However, the exact site of binding of chloride anions to hemoglobin was not known until very recently (2). However, these studies did not address the fundamental question of the mode in which chloride reduced the oxygen affinity of hemoglobin. We have felt that an understanding of the mechanism by which any modulator reduces oxygen affinity would be of value in the design of a hemoglobin-based blood substitute. In order to approach this question experimentally, we felt that a small covalent modifier with a negatively charged moiety was desirable. Such a group is the carboxymethyl group (3). This moiety is small, stably bound to the protein, and possesses the desirable negative charge. However, the manner by which it could be introduced selectively at only amino groups was not apparent until recently. Since our earlier studies and those of other investigators (4-10) had shown that the amino groups of proteins were important in the binding of inorganic anions such as chloride , a carboxymethylating agent specific for amino groups was clearly desirable.

Since the carboxymethyl group with its negative charge is so small, we deemed it unlikely that the functional properties of the protein would be perturbed to any large extent. This assumption has been borne out experimentally (3, 8, 9). Therefore, we now had a

hemoglobin derivative with a *covalently attached small anion*, which might be analogous to the presence of chloride binding to hemoglobin and the subsequent lowering of the oxygen affinity.

Another feature of the carboxymethyl group that attracted us was the fact that it could be considered as a stable analog of the adduct formed when CO_2 binds to hemoglobin. The binding of CO_2 to hemoglobin is also known to lower the oxygen affinity in a similar manner as chloride (11). However, the derivative of CO_2 and hemoglobin is very difficult to isolate because of its lability, since the reaction between hemoglobin and CO_2 is reversible. If this were not so, it would be difficult for us to expire CO_2. However, the carboxymethylation of hemoglobin is irreversible and that derivative is very readily isolated by ion exchange chromatography because of its charge difference with unmodified hemoglobin. Therefore, studies on carboxymethylated hemoglobin may reveal some information on the sites of binding of CO_2.

We have worked out a procedure to obtain this derivative in proteins. We studied the various conditions of pH and ratios of reactants to hemoglobin to achieve preferential reaction at the amino terminal residues of hemoglobin and to minimize reaction at the ϵ-amino groups of lysine residues (3). Hedlund and his colleagues (12) also used reductive carboxymethylation by our procedure in an attempt to achieve a useful blood.

It is now generally appreciated that a lowering of the oxygen affinity of hemoglobin can be achieved either by the neutralization of the positive charges in the DPG cleft by chloride, or by the introduction of a negative carboxymethyl group at this site. We also found recently that the removal of a positive charge by acetylation also had the same effect (13). Therefore, in our studies this past year we have also studied the site-specific acetylating - methyl acetyl phosphate - and these results are reported below.

A hemoglobin based blood substitute must be prevented from dissociation into dimers and monomers. To achieve this chemical cross-linking has been used and several agents have been investigated in various laboratories. We have evaluated glycolaldehyde based on some earlier work that we had done on the cross-linking of ribonuclease with this agent. However, it

has never been completely clear whether a hemoglobin derivative with a low oxygen affinity would be prevented from dissociating into dimers because the dissociation constant of *deoxy* hemoglobin is so low. Therefore, in the past year we have tested whether the carboxymethyl hemoglobin derivative with a low oxygen affinity would dissociate and be cleared from the circulation. Then collaboration was with Dr. John Hess the at Letterman Army Institute of Research.

Another question whose answer is not known concerns the optimum molecular weight of a hemoglobin-based blood substitute. It is appreciated that human serum albumin, which is about 60,000 molecular weight, is not cleared from the circulation. It is not clear whether this is because of its isoelectric point or its molecular weight of 60,000 alone (as a single polypeptide chain) is adequate to keep it from being cleared from the circulation. Accordingly, we have tested other types of cross-linking agents that would produce hemoglobin cross-linked *within* a tetramer rather than *between* tetramers. We had shown last year that glycolaldehyde promotes both types of cross-linking (14).

EXPERIMENTAL METHODS

Hemoglobin Preparations - Whole blood from normal individuals was centrifuged at 2,000 g for 10 min at 4^0C. After removal of the supernatant plasma, the cells were washed three times with cold isotonic saline, lysed by addition of an equal volume of cold distilled water and dialyzed against 0.1 N NaCl. The concentration of hemoglobin was determined by its absorbance at either 420 or 540 nm. The hemoglobin was purified by DE-52 chromatography to remove minor and glycosylated hemoglobin components.

Carboxymethylation Reaction - Many of the procedures have been described in our early publication (3) as well as in the publications during the first two years of this contract (8, 9, 14). Sodium glyoxylate was obtained from Sigma and sodium cyanoborohydride was from

Aldrich. Sephadex G-100 and Sepharose 4B were products of Pharmacia. All other chemicals were reagent grade and of the highest purity available.

Reductive Carboxymethylation of Hemoglobin A - Dialyzed lysates containing hemoglobin A (0.5-1.5 mM) were treated with a 10 fold excess of sodium glyoxylate and a 20 fold excess of sodium cyanoborohydride at 25^0C in 50 mM potassium phosphate, pH 7.4, for 60 min. For experiments with liganded hemoglobin, either the oxy or the CO form was used. If no further reactions were to be performed, the carboxymethylated hemoglobin was saturated with CO and the excess starting materials were removed by gel filtration on Sephadex G-25 (2 x 25 cm) with 50 mM Tris-acetate buffer, pH 7.3 as the eluent. If further reaction (i.e., cross-linking) of deoxyhemoglobin was to be performed, CO was not used. However, the gel fitration was still carried out in order to remove $NaCNBH_3$.

The carboxymethylated hemoglobin isolated to date by the above procedure is made up of 75% of Hb tetramers carboxymethylated on either two or four NH_2-terminal groups (8, 9, 14). We tentatively attribute the more selective reaction of *deoxy* hemoglobin at the NH_2-terminal residues compared with *oxy* hemoglobin as due to the fact that the amino terminal residues of hemoglobin change their pKas as a function of the state of oxygenation and the glyoxylate anion may then bind more efficiently.

Cross-Linking of Hemoglobin - Unmodified hemoglobin was used for these studies. The final concentration of hemoglobin was about 50 μM in 50 μM potassium phosphate buffer, pH 7.3. For studies with liganded hemoglobin, the CO form was used. For experiments with deoxyhemoglobin, the oxygen was removed by flushing with nitrogen as described above. The cross-linking agents that are undergoing testing are a series of diisothiocyante derivatives. These were added to the hemoglobin in different ratios. The cross-linking is performed at room temperature for varying times and the hemoglobin derivative is then dialyzed extensively against 50 M Tris-acetate, pH 7.3 to remove cross-linking reagent.

Progress of Cross-Linking Reaction - In general, the degree of cross-linking has been monitored by several techniques - SDS gel electrophoresis, gel electrophoresis under non-

denaturing conditions (Beckman Paragon system), column chromatography on DE-52 and gel filtration in the presence of 1 M $MgCl_2$.

Oxidation of Hemoglobin Derivatives - The conversion of ferro to ferric (Met) hemoglobin was determined by the amount of cyanmet hemoglobin. The oxidation was followed as a function of time at several temperatures as described below. Unmodified or carboxymethylated hemoglobin were tested for their respective rates of oxidation.

RESULTS AND DISCUSSION

Stability of Unmodified and of Carboxymethylated Hemoglobin - This past year we have conducted studies on the stability of carboxymethylated hemoglobin by measuring the rate of met hemoglobin formation at different temperatures. The sample that was used for this study was purified on DE-52 chromatography by methods reported previously (8, 9, 14). We found that for a period of several months carboxymethylated hemoglobin (not cross-linked) forms little if any detectable amount of met (oxidized) hemoglobin either at 4^0C or at -80^0C. However, at -20^0C there is greater oxidation of hemoglobin than at the other two temperatures. These results are shown in Fig 1. Under the same conditions oxygenated unmodified hemoglobin is oxidized to met hemoglobin in a short period of time. It is clear then from these results that the carboxymethylated hemoglobin is a somewhat more stable protein than unmodified hemoglobin.

Physiological Clearance Studies with Carboxymethylated Hemoglobin - In a collaborative study with Dr. John Hess and his colleagues of the Letterman Army Institute of Research we have determined the retention time of carboxymethylated hemoglobin (not cross-linked). This sample of carboxymethylated hemoglobin was purified by passage through a column of DE-52 cellulose to remove any unmodified hemoglobin and also the small amounts of hemoglobin components that were modified on their lysine residues. The sample contained only two types of hemoglobin tetrameric species - one of which contained two carboxymethylated

groups per tetramer and the other component contained four carboxymethylated groups per tetramer (14). We already know from previous analytical determinations that the carboxymethyl groups are on the N-terminal residues of either the α-chain or the β-chains (3).

Before the sample was sent to Dr. Hess, we removed any pyrogens present by passing the sample through a column of Detoxigel (Pierce). For the preparation of the hemoglobin derivative, the water that we used was prepared by reverse osmosis and was found to be pyrogen free by the limits of detection of the limulus assay. The sample was then shipped to Dr. Hess on ice by overnight express. Dr. Hess and his colleagues determined on their Hemox instrument that the sample of carboxymethylated hemoglobin had a P_{50} of 30 mm of Hg. This is the same value that we obtained on a carboxymethylated sample that we had prepared earlier under the same conditions. The hemoglobin derivative was then infused into six rats at a concentration of 13 gms/dL and into six other rats at a concentration of 6 gms/dL. The retention time of the samples was measured in each set of rats and it was calculated that the sample was cleared with a half-life of about 42 min (Fig. 2). Dr. Hess noted that this was about the same order of magnitude as the clearance of unmodified hemoglobin. The kinetics of clearance for the retention times were first order and these data are consistent with the conclusion that hemoglobin behaves as a homogeneous population of molecules. In addition Dr. Hess noted that there was no adverse pathological findings at autopsy.

We concluded from these studies that even though this hemoglobin derivative had a very low P_{50} there is a necessity for cross-linking in order to prevent its premature removal from circulation.

Analytical Procedures to Determine Whether Cross-Linking is Inter-Tetrameric or Intra-Tetrameric - In work performed last year under this contract we showed that when cross-linking is between subunits in the same tetramer, i.e., intra-tetrameric, then the degree of such cross-linking could be ascertained by gel filtration on Sephadex G-100. We found that the relative distribution of 64,000 molecular weight or higher molecular weight species was a function of the hemoglobin concentration used initially. Thus, if a dilute hemoglobin solution

was used (50 μM) then most of the cross-linking was within a hemoglobin tetramer. However, if the concentration of hemoglobin was about 10-fold higher then a significant fractions of the cross-linking was *between* hemoglobin tetramers (14).

In the past contract year we have also employed gel filtration on Sephadex but with a high concentration (1 M) of magnesium chloride in the eluent. This procedure, which has been described before by other investigators, permits one to separate non cross-linked tetramers from tetramers that are cross-linked between subunits and from tetramers that are cross-linked to one another. As shown in Fig. 3 the separation is very good. We have found this column to be especially useful with preliminary studies on the new cross-linking agents described below.

Preliminary Studies on New Cross-Linking Agents - During the last few months of this contract year we have initiated studies on a family of cross-linking agents of the diisocyanate family with the hope of producing primarily or exclusively intra-tetrameric cross-linked species of 64,000 molecular weight. The preliminary studies show that the cross-linking is quite efficient. Thus, we have studied the ratio of reactant to hemoglobin in a series of increasing ratios and we have found that a ratio of 1:10 of hemoglobin:cross-linker is an efficient ratio. SDS gel electrophoresis indicates that only cross-linked subunits of molecular weight 32,000 are found and there are no higher cross-linked species are detected.

In a series of studies just completed we have found that the pH optimum for this cross-linking reaction to maintain discrete species on a non-denaturing gel is between 7.2 and 7.5. For this purpose we use a rapid screening procedure using a native gel electrophoresis performed on the Beckman Paragon system.

We also find that when this cross-linked hemoglobin is applied to the Sephadex G-100 column in the presence of 1 M magnesium chloride then practically all of the material is of molecular weight 64,000. There are no higher cross-linked species. The degree of cross-linking is not effected by protein concentration. This latter result is consistent with the results from gel filtration in the presence of 1 M $MgCl_2$ that the major and perhaps the only cross-linking is within a hemoglobin tetramer and therefore not concentration dependent. This result is also

consistent with the earlier finding that glycolaldehyde, which is capable of produced 64,000 and greater molecular weight cross-linked species, does demonstrate a concentration dependence of the type of cross-linking on the initial hemoglobin concentration (14). Studies with this new type of cross-linker are continuing.

Three-Dimensional Structure of Cross-Linked Hemoglobins - In the past few months we have initiated studies on these new cross-linked derivatives with Dr. John Kuriyan of this institution. We are supplying Dr. Kuriyan with different samples of cross-linked hemoglobin. We expect that this information together with the peptide mapping that we intend to do will aid us in the identification of the site of the cross-link.

Studies with other Agents that Lower the Affinity of Hemoglobin - We had been investigating the compound, methyl acetyl phosphate, during the course of this contract and have found that the hemoglobin treated with this compound has some properties that are desirable of hemoglobin based blood substitute. We have found previously that methyl acetyl phosphate binds to the DPG cavity of deoxyhemoglobin preferentially and acetylates three amino groups in that region of the molecule (13). These are Val-1(β), Lys-82(β), and Lys-144(β). The effect on hemoglobin oxygen affinity is shown in Fig. 4. We have also done some experiments comparing the effect of the agent on human and bovine hemoglobin. Bovine hemoglobin utilizes chloride instead of DPG for lowering its oxygen affinity and it has more chloride binding sites than human hemoglobin. We find that MAP reacts more extensively with bovine hemoglobin than with human hemoglobin. This result is consistent with the finding that bovine hemoglobin is more responsive to chloride in lowering its oxygen affinity than is human hemoglobin. We have found that treatment of either human or bovine hemoglobins with methyl acetyl phosphate under anaerobic conditions has the same effect on the oxygen affinity as the covalent modifiers such as 2,3-DPG and chloride. This Hill coefficient of the hemoglobin is not affected by treatment with MAP. The results show that the effect of acetylation and chloride binding are mutually exclusive. Thus, for hemoglobin acetylated in either the oxy or deoxy conformation there is no further lowering of the oxygen affinity by the presence of

chloride. Furthermore, the degree of lowering of the oxygen affinity by chloride is the same as that obtained by saturating amounts of chloride. This result indicates that since chloride did not further lower the oxygen affinity of acetylated hemoglobin then the binding sites of chloride and bovine hemoglobin had already been acetylated by methyl acetyl phosphate. Thus, we conclude the presence of acetyl groups on certain amino groups blocks the binding of the anions chloride and DPG.

We are also able to identify some of the chloride binding sites in bovine hemoglobin by peptide mapping after treatment with radiolabeled methyl acetyl phosphate. These binding sites are Met-1(β) and Lys-81(β) for liganded hemoglobin and Val-1(α) and Lys-81(β) for the deoxy bovine hemoglobin. Thus, even though MAP acetylates the α-chain of deoxy bovine hemoglobin it does not acetylate the Val-1(α) of human hemoglobin. Since the structure of bovine hemoglobin is not currently known we speculate that the chloride binding site of Val-1(α) could be more accessible to solvent. Perhaps the reason that MAP does not acetylate Val-1(α) of human hemoglobin might be the tightness of charges in this area of the molecule.

Other Studies During the Past Contract Year - We also found that chloride in the concentrations usually used in many laboratories for experiments on hemoglobin in the presence of phosphate - buffered saline can have a signficant effect on the final pH. Thus, in very dilute phosphate buffer, the presence of chloride lowers the pH of this buffer by about 0.4 pH units (Fig. 5). Other buffers such as Tris acetate are also affected but the changes are much smaller in magnitude (Fig. 6). These findings indicate that whenever studies are performed on unmodified hemoglobin with chloride present in the buffers the pH must be rigorously controlled. In earlier studies with Wendy Fantl when she was a student in this laboratory we found that the leakage of chloride from certain electrodes was very significant (8, 9). These studies also point to the care that must be taken in the interpretation of results when freely diffusible anions that are capable of binding to hemoglobin are present.

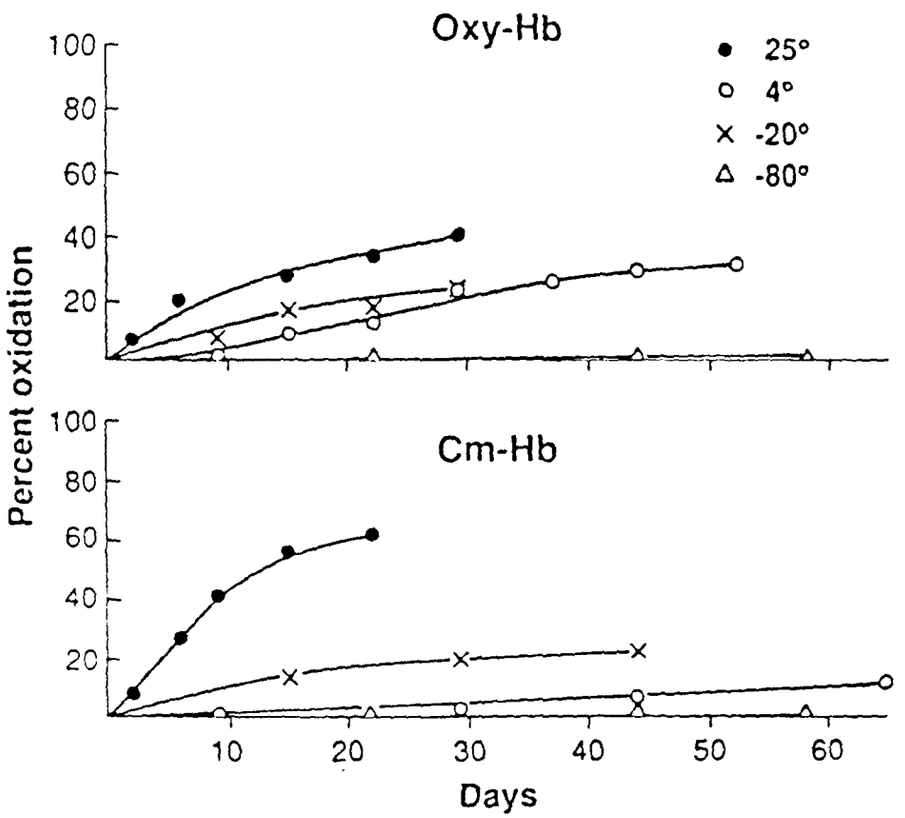

Fig. 1 - Stability of Oxy Hemoglobin and Cm-Hemoglobin at Different Storage Temperatures. The amount of met hemoglobin was determined as the cyanmet derivative.

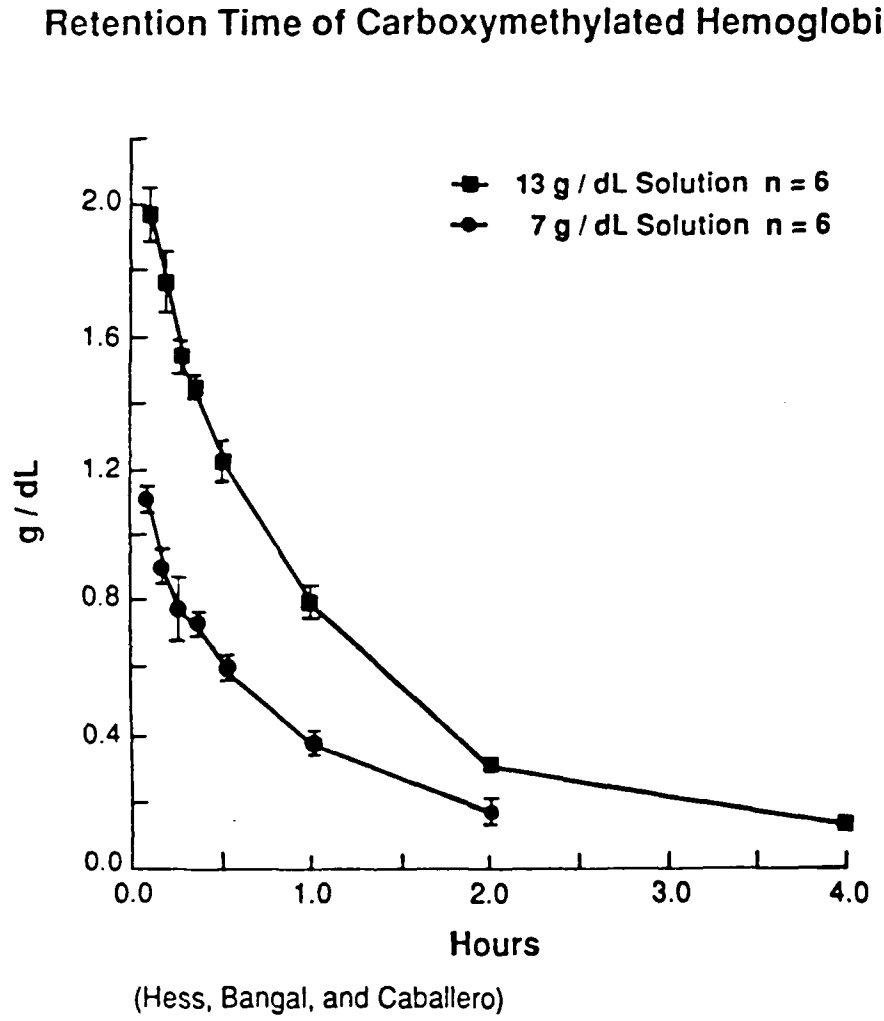

Fig. 2 - Clearance Time of Cm-Hemoglobin from the Circulation of Rats. These studies were performed by Dr. John Hess at Letterman Army Institute of Research on the hemoglobin derivative prepared by us.

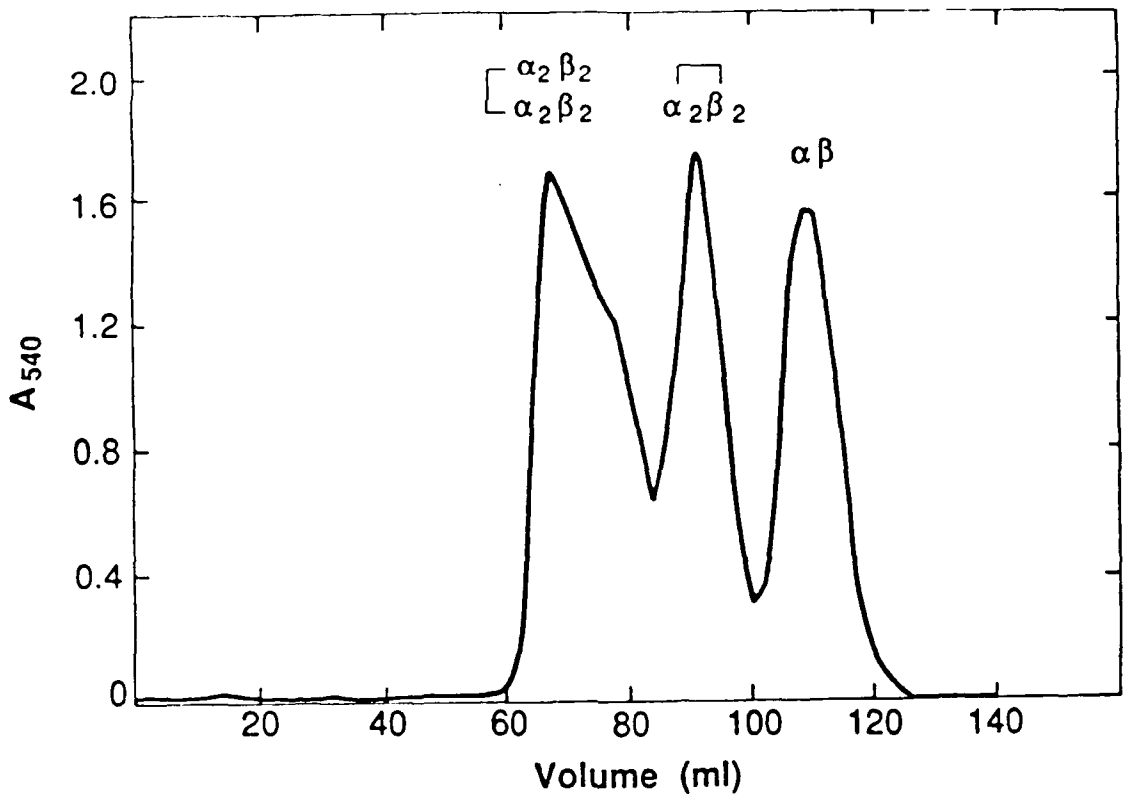

Fig. 3 - Separation of Cross-Linked Hemoglobin on G-100 Sephadex in High $MgCl_2$. The first peak contains species cross-linked *between* tetramers. The second peak contains species cross-linked *within* tetramers. The third peak represents uncross-linked dimers.

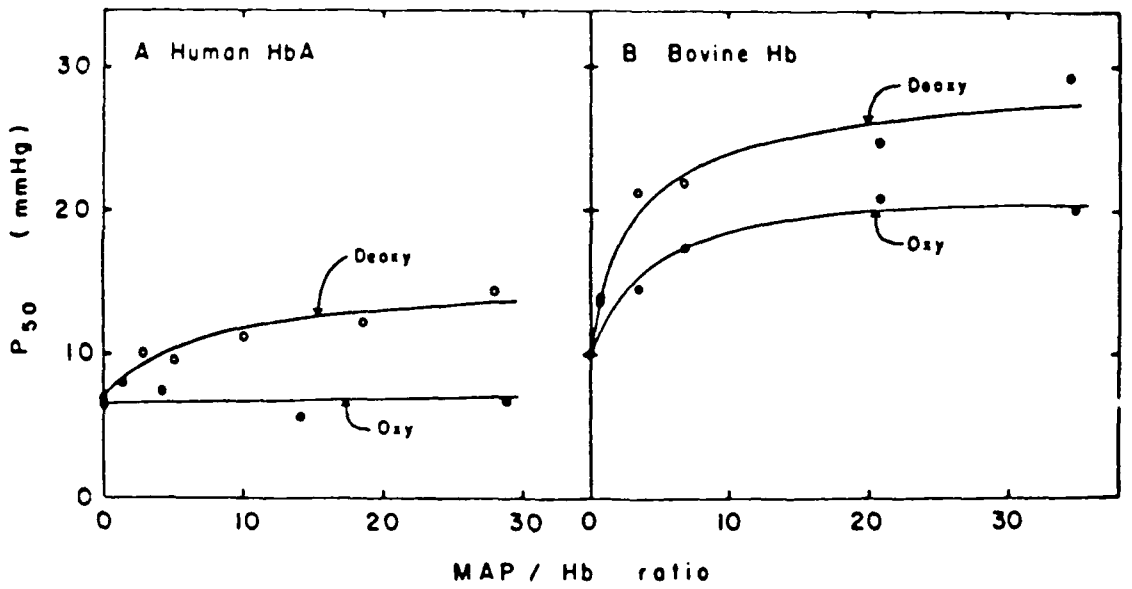

Fig. 4 - Effect of Methyl Acetyl Phosphate (MAP) on the Oxygen Affinity of Human and Bovine Hemoglobin.

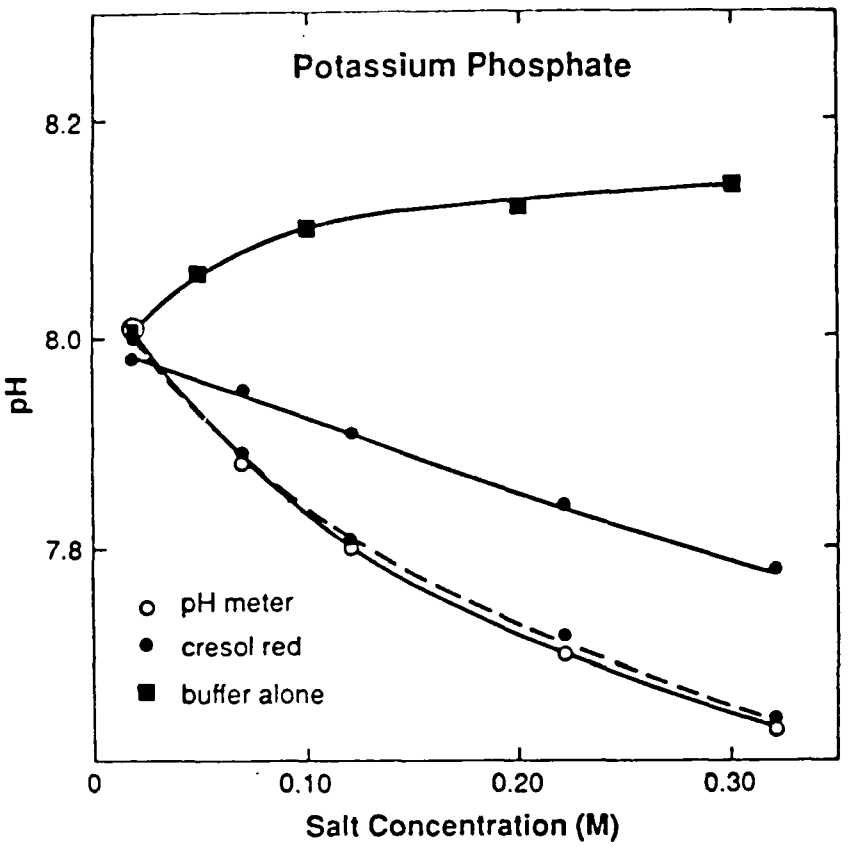

Fig. 5 - Effect of Chloride on the pH of Phosphate Buffer.

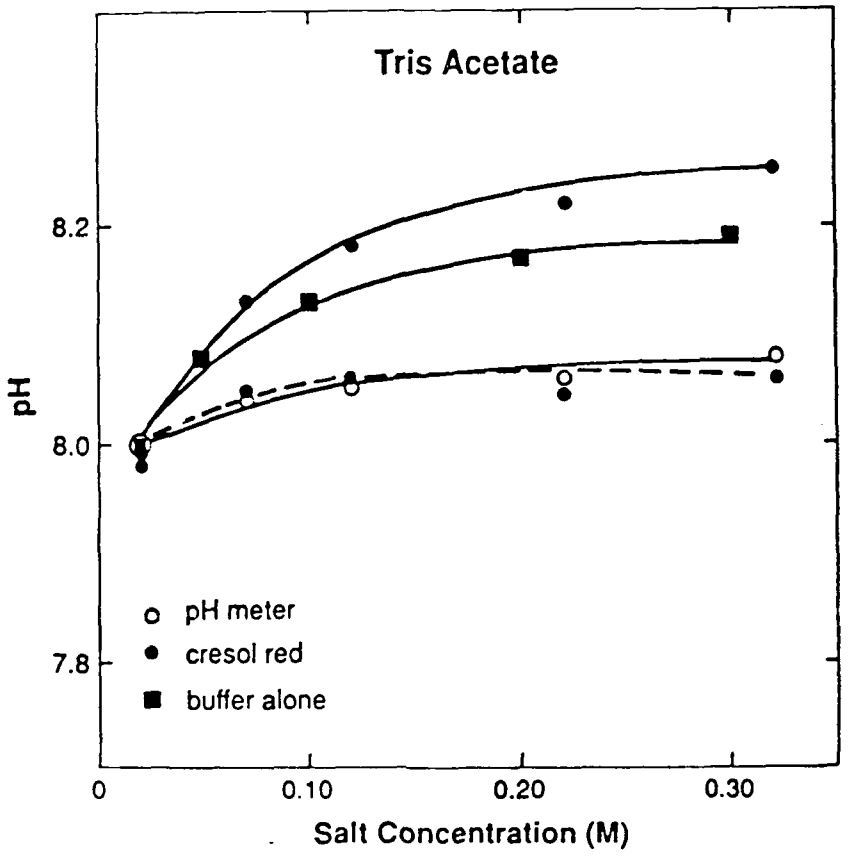

Fig. 6 - Effect of Chloride on the pH of Tris-Acetate Buffer.

REFERENCES

1. Antonini, E., and Brunori, M. (1971) in "Hemoglobin and Myoglobin in Their Reactions with Ligands", Elsevier, New York.

2. Chiancone, E., Norne, J.E., Forsen, S., Bonaventura, J., Brunori, M., Antonini, E., and Wyman, J. (1975) *Eur. J. Biochem.* 55:385-390.

3. Di Donato, A., Fantl, W.J., Acharya, A.S., and Manning, J.M. (1983) *J. Biol. Chem.* 258:11890-11895.

4. Nigen, A. M., and Manning, J. M. (1975) *J. Biol. Chem.* 250:8248-8250.

5. Nigen, A. M., Manning, J. M., and Alben, J. O. (1980) *J. Biol. Chem.* 255:5525-5529.

6. Manning, J.M., Nigen, A.M., Charache, S. and Alben, J.O. (1978) in "Biochemical and Clinical Aspects of Hemoglobin Abnormalities", W.S. Caughey (ed), Academic Press, New York, 687-694.

7. O'Donnell, S., Mandavo, R., Schuster, T.M., and Arnone, A. (1979) *J. Biol. Chem.* 254:12204-12208.

8. Fantl, W. J., Di Donato, A., Manning, J. M., Rogers, P. H., and Arnone, A. (1987a) *J. Biol. Chem.* 26:12700-12713.

9. Fantl, W. J., Manning, L. R., Ueno, H., Di Donato, A., and Manning, J. M. (1987b) *Biochemistry* 26:5755.

10. Rollema, H.S., DeBruin, S.H., Janssen, L.H.M., and Van Os A.J. (1975) *J. Biol. Chem.* 250:1333-1339.

11. Kilmartin, J.V., Fogg, J., Luzzana, M., and Rossi-Bernardi, L. (1973) *J. Biol. Chem.* 248:7039-7043.

12. Saunders, S., and Hedlund, B.E. (1984) *Biochemistry* 23:1457-1461.

13. Ueno, H., Pospischil, M.A., and Manning, J.M. (1989) *J. Biol. Chem.* 264:12344-12351.

14. Manning, L.R., and Manning, J.M. (1988) *Biochemistry* 27:6640-6644.